(For Laboratory Use Only)
ATS Labs Project # A12007
LC 9/ac/11

ATS LABS

PROTOCOL

Efficacy of a Disinfectant Applied Via an Ultra Low Volume Misting Device

Test Organisms:

Pseudomonas aeruginosa (ATCC 15442)
Staphylococcus aureus (ATCC 6538)
Salmonella enterica (ATCC 10708)

PROTOCOL NUMBER

ZIM01022811.CUST.1

PREPARED FOR

Zimek Technologies LLC
9328 Florida Palm Drive
Tampa, FL 33619

PERFORMING LABORATORY

ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

PREPARED BY

Amy S. Jeske, B.S.
Research Scientist I

DATE

February 28, 2011
Revised Date: July 26, 2011

EXACT COPY
INITIALS BS DATE 9/7/11

1285 Corporate Center Drive, Suite 110 • Eagan, MN 55121 • 877.287.8378 • 651.379.5510 • Fax: 651.379.5549

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Efficacy of a Disinfectant Applied Via an Ultra Low Volume Misting Device

SPONSOR: Zimek Technologies LLC
9328 Florida Palm Drive
Tampa, FL 33619

TEST FACILITY: ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

PURPOSE

The purpose of this assay is to determine the efficacy of an EPA registered disinfectant on exposed, hard, non-porous surfaces, which is applied through an ultra low volume misting device into a sealed enclosure.

TEST SUBSTANCE CHARACTERIZATION

Test substance characterization as to content, stability, etc., (40 CFR, Part 160, Subpart F [160.105]) is the responsibility of the Sponsor. The test substance shall be characterized by the Sponsor prior to the experimental start date of this study. Pertinent information, which may affect the outcome of this study, shall be communicated in writing to the Study Director upon sample submission to ATS Labs.

SCHEDULING AND DISCLAIMER OF WARRANTY

Experimental start dates are generally scheduled on a first-come/first-serve basis once ATS Labs receives the Sponsor approved/completed protocol, signed fee schedule and corresponding test substance(s). Based on all required materials being received at this time, the proposed experimental start date is August 15, 2011. Verbal results may be given upon completion of the study with a written report to follow on the proposed completion date of September 2, 2011. To expedite scheduling, please be sure all required paperwork and test substance documentation is complete/accurate upon arrival at ATS Labs.

If a test must be repeated, or a portion of it, due to failure by ATS Labs to adhere to specified procedures, it will be repeated free of charge. If a test must be repeated, or a portion of it, due to failure of internal controls, it will be repeated free of charge. "Methods Development" fees shall be assessed, however, if the test substance and/or test system require modifications due to complexity and difficulty of testing.

If the Sponsor requests a repeat test, they will be charged for an additional test.

Neither the name of ATS Labs or any of its employees are to be used in advertising or other promotion without written consent from ATS Labs.

The Sponsor is responsible for any rejection of the final report by the regulatory agencies of its submission concerning report format, pagination, etc. To prevent rejection, Sponsor should carefully review the ATS Labs final report and notify ATS Labs of any perceived deficiencies in these areas before submission of the report to the regulatory agency. ATS Labs will make reasonable changes deemed necessary by the Sponsor, without altering the technical data.

JUSTIFICATION FOR SELECTION OF THE TEST SYSTEM

Regulatory agencies require that specific claims for a disinfectant product applied through an application system intended for the disinfection of inanimate surfaces be supported by appropriate scientific data demonstrating the efficacy of the product and delivery system against the claimed test organism. This is accomplished in the laboratory by treating the test organism with the product (test substance) under conditions which simulate as closely as possible the actual conditions under which the test substance is designed to be used. For ultra low volume misting devices which utilize a registered disinfectant, efficacy testing is performed to determine that all exposed, hard, non-porous surfaces within the enclosure are effectively treated with the disinfectant. The experimental design in this protocol meets these requirements. This protocol has not been reviewed by regulatory agencies for registration compliance. Acceptance of this protocol by a regulatory agency is the responsibility of the Sponsor.

TEST PRINCIPLE

Inoculated glass carriers are placed at diverse locations within a sealed enclosure. The inoculated carriers will be exposed to the test substance for a specified exposure time. After exposure, the carriers are transferred to vessels containing neutralization / subculture media and assayed for survivors. Appropriate culture purity, viability, media sterility, carrier sterility, organic soil sterility, neutralization confirmation and carrier population controls will be performed.

TEST METHOD

Table 1:

Test Organisms	ATCC #	Growth Medium	Incubation Parameters
Pseudomonas aeruginosa	15442	Nutrient Broth	35-37°C, aerobic
Staphylococcus aureus	6538	Synthetic Broth	35-37°C, aerobic
Salmonella enterica	10708	Synthetic Broth	35-37°C, aerobic

The test organisms to be used in this study were obtained from the American Type Culture Collection (ATCC), Manassas, VA.

Preparation of the Sealed Enclosure

For Enclosures >1250 feet^3
A test room enclosure with dimensions 18'8.5" x 16'8" x 11'9", totaling 3663.7 feet^3 will be used for testing when the application system operates within a sealed enclosure.

For Enclosures <1250 feet^3
A test room enclosure with a volume of <1250 feet^3 will be used for testing when the application system is placed outside the sealed enclosure allowing the ULV mist to be ducted into the space from a single entry point. In this configuration, ducting provided by the Sponsor will be fitted to a suitable opening into the enclosure and connected to the application system.

not needed per client conversation 8/25/11

In either case, the enclosure will be prepared by removing all visible dirt and debris. This may involve sweeping and mopping the floor and washing all visibly dirty surfaces. Shelves or other suitable means of supporting the test carriers will be positioned on walls at different heights. The enclosure will be sealed prior to testing in order to isolate the space in which the test substance will be applied. At a minimum, this will include sealing the door to the room by any appropriate means. The HVAC vents in the enclosure may also be covered. Environmental conditions will be monitored prior to, during testing and at reentry. Supplemental fans may be used to assist with distribution of the ULV mist at the Sponsor's request.

Preparation of Test Carriers

Glass slides (1 inch x 1 inch or 18 mm x 36 mm) will be utilized as the carrier for this assay. The carriers will be placed into a vessel and sterilized in an air oven for ≥2 hours at ≥180°C. Individual sterile plastic Petri dishes will be matted with two pieces of filter paper. Multiple glass slides will be transferred into each of the matted Petri dishes.

The minimum number of test carriers needed will be determined by the equation below.

Test carriers = $[(m^3\text{-}10) / 2] + 15$

Where m^3 = the volume of the room enclosure in cubic meters

When using a sealed enclosure with dimensions of 18'8.5" x 16'8" x 11'9", a minimum of 62 test carriers per organism will be used. When using a sealed enclosure with alternate dimensions the number of carriers to be used per organism will be calculated accordingly. Additional carriers may be utilized at Sponsor's request to determine the test substances ability to treat elevated ceilings.

Preparation of Test Organisms
From a stock slant, an initial tube of culture broth will be inoculated. This culture is termed the "initial broth suspension." From this initial broth suspension, a minimum of three daily transfers will be performed on consecutive days prior to use in testing procedure. For each test organism, the appropriate growth medium will be subcultured using a daily transfer (more than 3, but less than 30 transfers) of the test organism.

A 48-54 hour broth culture incubated at the parameters listed in Table 1 will be prepared.

On the day of use, the pellicle will be aspirated from the *Pseudomonas aeruginosa* culture. The test cultures will be thoroughly mixed and allowed to stand for ≥10 minutes prior to use.

An organic soil load may be added to the test culture per Sponsor's request.

Contamination of Carriers
The glass slide carriers will each be inoculated with 10.0 µL of a prepared suspension (using a 4 mm loop or calibrated pipettor) uniformly spreading the culture over the entire surface of the slide. The dish will be covered immediately and the procedure repeated until all slides have been individually inoculated. The slides will be allowed to dry for 30-40 minutes at 35-37°C. The drying conditions (temperature and humidity) will be appropriate for the test organism for the purpose of obtaining maximum survival following drying. The actual drying conditions will be clearly documented.

Carrier Placement in the Room Enclosure
Inoculated and dried test carriers will be placed within the room to include at a minimum:

1. All corners of the room
2. Central locations on all wall faces
3. Central locations on the floor
4. Underneath horizontal surfaces
5. Multiple locations and heights within the enclosed space

Carriers will be placed in near vertical and horizontal positions throughout the enclosure. In order to assess the efficacy of the disinfectant at typical ceiling heights, 44 carriers will be placed throughout the room at heights below 8 feet, 18 carriers will be placed throughout the testing room at a height of approximately 8 feet from the testing room floor and an additional 18 carriers will be placed throughout the testing room at a height of approximately 11 feet from the testing room floor. The locations of test carriers, vents, doors, application equipment, and entry duct (if used) will be documented. See protocol attachment for positioning diagram.

Test Substance and Equipment Preparation
The test substance(s) to be assayed will be used as directed by the Sponsor. If a dilution of the test substance is requested by the Sponsor, the test substance(s) will be diluted by ATS Labs and will be applied within three hours of dilution. The appropriate test substance volume will be added to the Sponsor provided application equipment and will be activated per Sponsor's instruction. Alternately, the Sponsor or Sponsor Representative may be present on the day of testing to prepare and activate the disinfectant application equipment.

Exposure
Once each of the inoculated carriers is positioned and the required environmental conditions are achieved, the lid of each Petri plate containing the inoculated carrier will be removed. The disinfectant application unit will be activated, the technician will immediately exit the room, and the room will be sealed. The exposure will continue for the duration specified by the Sponsor. The total time from activation to the completion of cycle will be documented.

Following the completion of the test cycle, the room may be aerated as indicated by the Sponsor in order to assure the technicians may safely enter the room. If an aeration step is required, the total aeration time will be recorded.

Test System Recovery
After exposure to the test substance, the treated test carriers will be individually transferred using sterile forceps to 20 mL aliquots of neutralizing subculture medium. If necessary, carriers will be transferred into individual secondary subcultures containing 20 mL aliquots of neutralizing medium ≥30 minutes after subculture of first carrier.

Incubation and Observation
All subcultures and controls are incubated for 48±4 hours at 35-37°C (or other appropriate time/temperatures). Following incubation, the subcultures will be visually examined for growth. If necessary, the subcultures may be placed at 2-8°C for up to three days prior to examination.

Representative neutralized subcultures showing growth will be subcultured, stained and/or biochemically assayed to confirm or rule out the presence of the test organism.

STUDY CONTROLS

Purity Control
A "streak plate for isolation" will be performed on the organism culture and following incubation examined in order to confirm the presence of a pure culture. The acceptance criterion for this study control is growth of a pure culture demonstrating colony morphology typical of the test organism.

Organic Soil Sterility Control
If applicable, the serum used for soil load will be cultured, incubated, and visually examined for lack of growth. The acceptance criterion for this study control is lack of growth.

Carrier Sterility Control
A representative uninoculated carrier will be added to the neutralizing subculture medium. The subculture medium containing the carrier will be incubated and examined for growth. The acceptance criterion for this study control is lack of growth.

Neutralizing Subculture Medium Sterility Control
A representative sample of uninoculated neutralizing subculture medium will be incubated and visually examined. The acceptance criterion for this study control is lack of growth.

Viability Control
A representative inoculated carrier will be added to the subculture medium. The subculture medium containing the carrier will be incubated and visually examined for growth. The acceptance criterion for this study control is growth.

Neutralization Confirmation Control
The neutralization of the test substance will be confirmed by exposing sterile carriers (representing not less than 10% of the total number of test carriers) to the test substance concurrently with the testing and transferring them to primary subcultures containing 20 mL of neutralizing subculture medium. If performed in the test procedure, carriers will then be transferred from primary subcultures into individual secondary subcultures ≥30 minutes following the primary transfer. The subcultures containing the exposed carriers will be inoculated with ≤100 colony forming units (CFU) of each test organism, incubated under test conditions and visually examined for the presence of growth. This control will be performed with multiple replicates using different dilutions of the test organism. A standardized spread plate procedure will be run concurrently in order to enumerate the number of CFU actually added. The control result will be reported using data from the most appropriate dilution.

The acceptance criterion for this study control is growth following inoculation with ≤100 CFU.

OR:

Ten percent of the subcultures containing carriers showing no growth will be inoculated with ≤100 CFU of each test organism and incubated. This control will be performed with multiple replicates representing different dilutions of the test organism. A standardized spread plate procedure will be run concurrently in order to enumerate the number of CFU actually added. The control result will be reported using data from the most appropriate dilution.

The acceptance criterion for this study control is growth following inoculation with ≤100 CFU.

Pre-Treatment Carrier Population Control
This control will be performed immediately following carrier placement in the testing room (Time zero). Inoculated carriers will be added at a ratio of 1 carrier to 10 mL neutralizing broth and vortex mixed. Appropriate serial ten-fold dilutions will be prepared and the aliquots spread plated on agar plate medium, and incubated. Following incubation, the resulting colonies will be enumerated and the CFU/carrier calculated. The acceptance criterion for this study control is a minimum of 1.0×10^4 CFU/carrier for the Time Zero population control.

Post-Treatment Carrier Population Control
Inoculated carriers will be placed within a sealed container and held in the test room during the test substance application. Upon completion of the treatment cycle the carriers will be removed from the sealed container. The carriers will be added at a ratio of 1 carrier to 10 mL neutralizing broth and vortex mixed. Appropriate serial ten-fold dilutions will be prepared and the aliquots spread plated on agar plate medium, and incubated. Following incubation, the resulting colonies will be enumerated and the CFU/carrier calculated. This control is performed for informational purposes only in order to determine the viable number of organisms on the test carriers following the treatment time. Therefore, this control has no acceptance criterion.

PROCEDURE FOR IDENTIFICATION OF THE TEST SYSTEM
ATS Labs maintains Standard Operating Procedures (SOPs) relative to efficacy testing studies. Efficacy testing is performed in strict adherence to these SOPs which have been constructed to cover all aspects of the work including, but not limited to, receipt, log-in, and tracking of biological reagents including bacterial strains for purposes of identification, receipt and use of chemical reagents. These procedures are designed to document each step of efficacy testing studies. Appropriate references to media, batch numbers, etc. are documented in the raw data collected during the course of each study.

Additionally, each efficacy test is assigned a unique Project Number when the protocol for the study is initiated by the Study Director. This number is used for identification of the test subculture tubes, etc. during the course of the test. Test subculture tubes are also labeled with reference to the test organism, experimental start date, and test product. Microscopic and macroscopic evaluations of positive subcultures are performed in order to confirm the identity of the test organism. These measures are designed to document the identity of the test system.

STUDY ACCEPTANCE CRITERIA

Test Substance Performance Criteria
The U.S. EPA efficacy performance requirements for label claims state that the test substance must kill the microorganisms on 59 out of 60 inoculated carriers (or the equivalent ratio if a different number of carriers are used).

Due to the Sponsor's desire to determine the extreme application limits of the test substance, multiple label claim options exist.

> For instance, if the test organism is killed on a minimum of 79 out of 80 inoculated test carriers placed at 11 feet or below from the testing room floor, then the label claims will be limited to applications in rooms with ceiling heights of 11 feet or less.
>
> Alternately, if the test organism is killed on a minimum of 61 out of 62 inoculated test carriers placed at 8 feet or below from the testing room floor, then the label claims will be limited to applications in rooms with maximum ceiling heights of 8 feet.

Control Acceptance Criteria
The study controls must perform according to the criteria detailed in the study controls description section. If any of the control acceptance criteria are not met, the test may be repeated under the current protocol number.

METHOD FOR CONTROL OF BIAS: N/A

REPORT
The report will include, but not be limited to, identification of the sample, date received, initiation and completion dates, identification of the bacterial strains used, description of media and reagents, description of the methods employed, tabulated results and conclusion as it relates to the purpose of the test, and all other items required by 40 CFR Part 160.185.

PROTOCOL CHANGES

If it becomes necessary to make changes in the approved protocol, the revision and reasons for changes will be documented, reported to the Sponsor and will become a part of the permanent file for that study. Similarly, the Sponsor will be notified as soon as possible whenever an event occurs that may have an effect on the validity of the study. Standard operating procedures used in this study will be the correct effective revision at the time of the work. Any minor changes to SOPs (for this study) or methods used will be documented in the raw data and approved by the Study Director.

TEST SUBSTANCE RETENTION

It is the responsibility of the Sponsor to retain a sample of the test substance. All unused test substance will be discarded following study completion unless otherwise indicated by Sponsor.

RECORD RETENTION

Study Specific Documents

All of the original raw data developed exclusively for this study shall be archived at ATS Labs. These original data include, but are not limited to, the following:

1. All handwritten raw data for control and test substances including, but not limited to, notebooks, data forms and calculations.
2. Any protocol amendments/deviation notifications.
3. All measured data used in formulating the final report.
4. Memoranda, specifications, and other study specific correspondence relating to interpretation, and evaluation of data, other than those documents contained in the final study report.
5. Original signed protocol.
6. Certified copy of final study report.
7. Study-specific SOP deviations made during the study.

Facility Specific Documents

The following records shall also be archived at ATS Labs. These documents include, but are not limited to, the following:

1. SOPs which pertain to the study conducted.
2. Non study-specific SOP deviations made during the course of this study which may affect the results obtained during this study.
3. Methods which were used or referenced in the study conducted.
4. QA reports for each QA inspection with comments.
5. Facility Records: Temperature Logs (ambient, incubator, etc.), Instrument Logs, Calibration and Maintenance Records.
6. Current curriculum vitae, training records, and job descriptions for all personnel involved in the study.

REFERENCES

1. Association of Official Analytical Chemists (AOAC), 2006. Use-Dilution Methods 964.02, 955.14, and 955.15.
2. Association of Official Analytical Chemists (AOAC), 2005. Germicidal and Detergent Sanitizing Action of Disinfectants Method 960.09 [Preparation of Synthetic Hard Water].
3. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, 1982. Efficacy Data Requirements, Disinfectants for Use on Hard Surfaces, DIS/TSS-1.
4. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, 1979. Efficacy Data Requirements, Supplemental Recommendations, DIS/TSS-2.
5. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, Draft Protocol "Protocol for Sterilization of Porous and Non-Porous Surfaces within Sealed Enclosures using Vaporized Hydrogen Peroxide"
6. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, 1982. Subseries 91A: Public Health Uses. *In* Pesticide Assessment Guidelines – Subdivision G (Product Performance).

DATA ANALYSIS

Calculations

Carrier Quantitation Calculation:

$$\frac{\text{(avg. \# colonies /plate @ dilution used) (dilution factor) (volume subculture medium)}}{\text{(\# of carriers tested) (volume plated)}}$$

Statistical Methods

None used

Sept. 25, 2011

STUDY INFORMATION

(All sections must be completed prior to submitting protocol)

Sponsor (Date/Initial): JC, Sept. 25, 2011

Test Substance (Name & Batch Numbers, *including ≥60 day old batch* - exactly as it should appear on final report):
Vital Oxide Lot VORTU20110715

Specify ≥60 day old batch: Lot VORTU20110715

Expiration Date: 7/15/2012

Product Description:

- ☑ Quaternary ammonia
- ☐ Iodophor
- ☐ Peracetic acid
- ☐ Peroxide
- ☐ Sodium hypochlorite
- ☑ Other Chlorine Dioxide

Test Substance Active Concentration (upon submission to ATS Labs): 0.200% Chlorine Dioxide, 0.125% Quat

Neutralization/Subculture Broth:

- ☐ _____
- ☑ ATS Labs' Discretion. By checking, the Sponsor authorizes ATS Labs, at their discretion, to perform neutralization confirmation assays at the Sponsor's expense prior to testing to determine the most appropriate neutralizer. (See Fee Schedule).

Storage Conditions:

- ☑ Room Temperature
- ☐ 2-8°C
- ☐ Other: _____

Hazards:

- ☐ None known: Use Standard Precautions
- ☑ Material Safety Data Sheet, Attached for each product
- ☐ As Follows: _____

Product Preparation

- ☑ No dilution required, Use as received (RTU)
- ☐ *Dilution(s) to be tested:

_____ defined as _____ + _____
(example: 1 oz/gallon) (amount of test substance) (amount of diluent)

- ☐ Deionized Water (Filter or Autoclave Sterilized)
- ☐ Tap Water (Filter or Autoclave Sterilized)
- ☐ AOAC Synthetic Hard Water: _____ PPM
- ☐ Other _____

Note: An equivalent dilution may be made unless otherwise requested by the Sponsor.

Test Organism(s): *Pseudomonas aeruginosa* (ATCC 15442)
Staphylococcus aureus (ATCC 6538)
Salmonella enterica (ATCC 10708)

Carrier Number: 80/organism

Equipment Operational Instructions: To be operated by Sponsor

Exposure Time(s): 1 treatment cycle – actual time will be document in the raw data and final report

Exposure Environmental Conditions: Ambient
(Temperature range and minimum humidity)

Organic Soil Load:

- ☐ Minimum 5% Organic Soil Load (Fetal Bovine Serum)
- ☑ No Organic Soil Load Required
- ☐ Other: _____

TEST SUBSTANCE SHIPMENT STATUS

- ☐ Has been used in one or more previous studies at ATS Labs.
- ☑ Has been shipped to ATS Labs (but has not been used in a previous study).
  Date shipped to ATS Labs:_____ Sent via *overnight* delivery? ☐ Yes ☐ No
- ☐ Will be shipped to ATS Labs.
  Date of expected receipt at ATS Labs:_____
- ☐ Sender (if other than Sponsor):_____

COMPLIANCE

Study to be performed under EPA Good Laboratory Practice regulations (40 CFR Part 160) and in accordance to standard operating procedures.
☑ Yes
☐ No (Non-GLP Study)

PROTOCOL MODIFICATIONS

- ☐ Approved without modification
- ☑ Approved with modification - Supplemental Information Form Attached - ☑ Yes ☐ No

APPROVAL SIGNATURES

SPONSOR:

NAME: Mr. Jose Chavez TITLE: Consultant

SIGNATURE: _____ DATE: Sept. 25, 2011

PHONE: 813-506-2290 FAX: _____ EMAIL: Jschavez84@gmail.com

For confidentiality purposes, study information will be released only to the sponsor/representative signing the protocol (above) unless other individuals are specifically authorized in writing to receive study information.

Other individuals authorized to receive information regarding this study: ☐ See Attached

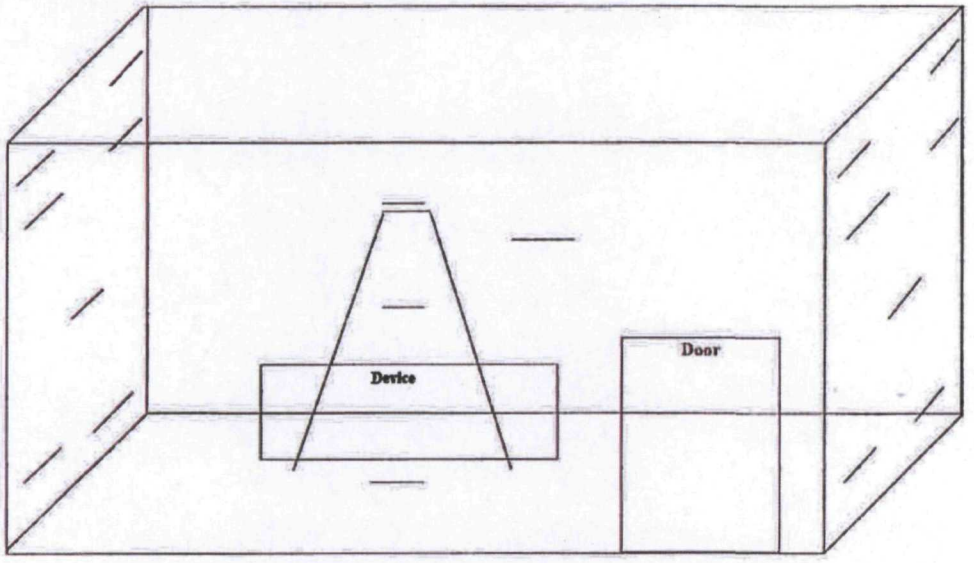
Kurt Grosman

ATS Labs:

NAME: Becky Liem
Study Director

SIGNATURE: _____ DATE: 9/26/11
Study Director

Attachment To ATS Labs Protocol ZIM01022811.CUST.1 –
Carrier Placement Diagram



Carriers will be placed on the horizontal lines throughout the room. Eighteen carriers will be distributed at both 8 feet and 11 feet heights. Forty four carriers will be distributed evenly amongst the areas below 8 feet. A three shelf laboratory cart, which is not included in the diagram above, will be located in the room and carriers will be placed on the middle and bottom shelves to represent locations under horizontal surfaces. These locations will be included in the calculation for determining how many carriers should be placed in each location below 8 feet.

Additional Shelves may be placed on adjacent walls to accommodate the number of carriers in testing.

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